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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re United States Patent Application of:		Customer No.:	23448
Applicant:	Von Knebel-Doeberitz, et al.	Docket No.:	4121-121
Serial No.:	09/719,336	Examiner:	C. Qian
Date Filed:	March 22, 2001	Art Group:	1633
Title:	USE OF ADENO-ASSOCIATED VIRUSES FOR DECREASING THE RADIOTHERAPY-INDUCED OR CHEMOTHERAPY-INDUCED RESISTANCE IN CANCER PATIENTS	Confirmation No.:	7154

EXPRESS MAIL CERTIFICATE

I hereby certify that I am mailing the attached documents to the Commissioner for Patents on the date specified below, in an Express Mail envelope addressed to the Commissioner for Patents, Box RCE, Washington, DC 20231, and Express Mailed under the provisions of 37 CFR 1.10.

Lauren E Ashe

Lauren Ashe

December 13, 2002

Date of Mailing

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DECLARATION OF DR. MAGNUS VON KNEBEL-DOEBERITZ IN U.S. PATENT APPLICATION NO. 09/719,336

Box RCE
Commissioner for Patents
Washington, D.C. 20231

Sir:

I, Magnus Von Knebel-Doeberitz, hereby declare:

1. THAT I am a named co-inventor of the invention that is described and claimed in U.S. Patent Application No. 09/719,336 filed in the United States Patent and Trademark Office on March 22, 2001 in the names of Magnus Von Knebel-Doeberitz, Petra Klein-Bauernschmitt, Harald Zur Hausen and Jorg Schlehofer for "USE OF ADENO-ASSOCIATED VIRUSES FOR DECREASING THE RADIOTHERAPY-INDUCED OR CHEMOTHERAPY-INDUCED RESISTANCE IN CANCER PATIENTS" (the "Application").

2. THAT the invention of the Application relates to the use of adeno-associated viruses for decreasing the radiotherapy-induced or chemotherapy-induced resistance in patients who suffer form a cancer which is treated by radiotherapy or chemotherapy.

3. THAT we have shown that AAV-2 has tumor-suppressive activity and sensitizes several human cancer cell lines and freshly explanted human tumor tissues to γ irradiation and to various chemotherapeutic agents *in vitro* and *in vivo*. Growth of tumor cells transplanted s.c. into nude mice was reduced more efficiently in AAV-2-infected animals than in noninfected animals, which were treated with the same chemotherapy or radiation regimen. Further we found that the mice infected with the AAV-2 virus and treated with chemotherapeutic drugs remained in better physical condition compared to controls treated with only chemotherapy. Also we investigated whether infection with AAV-2 might also enhance the cytotoxic effect of 5-FU on pancreatic tumor cells. The experiments described hereinbelow demonstrate that AAV infection prior to treatment with 5-FU compared to 5-FU treatment alone significantly reduced the viability of pancreatic cancer cells *in vitro* and reduced tumor growth in immunocompetent Lewis rats challenged with syngeneic pancreatic cancer cells. Furthermore, chemotherapy-related toxic side effects, such as thrombocytopenia, neutropenia, loss of weight and pain, were significantly reduced in animals treated with concomitant AAV infection.

4. THAT the following Methods and Material we used in the In Vivo testing procedures:

 <u>IN VIVO INFECTION</u> The human pancreatic cancer cell lines Capan-1 and DANG were provided by Tumorbank (DKFZ, Heidelberg, Germany). The murine tumor cell line DSL6A was originally derived from a primary pancreatic carcinoma of an azaserine-treated Lewis rat. All cells were grown in DMEM (Life Technologies, Karlsruhe, Germany) supplemented with 10% heat-inactivated FCS (Life Technologies), 100 units/ml penicillin and 100 µg/ml streptomycin (Life Technologies) and incubated at 37°C in a humidified atmosphere with 5% CO_2.

 AAV-2 was propagated in HeLa cells using adenovirus type 2 as a helper. Cells were lysed by 3 rounds of freezing and thawing, and AAV-2 virions were purified by CsCl gradient centrifugation as described previously. Infectious titers of AAV-

2 fractions were determined using end point titration (1:10 dilution steps in a volume of 100 µl) as described. Briefly, HeLa cells grown in 96-well plates were infected with 100 µl/well of each AAV-2 dilution and adenovirus was added as a helper [multiplicity of infection (MOI) 10 i.u./cell]. After complete cell lysis, the supernatant and remaining cells were mixed with 120 µl of 0.5 M NaOH and dotted onto a nylon membrane (Hybond N; Amersham, Freiburg, Germany). A ^{32}P-labeled EcoRI/HindIII fragment corresponding to the 3' part of the AAV genome (pTAV2[23]) was used as a probe for hybridization of the blots to determine at which dilution AAV-2 replication was still detectable.

Immunocompetent Male Lewis rats (Charles River, Sulzfeld, Germany) were kept in isolators and received food and water *ad libitum.* DSL6A tumor cells were injected s.c. into the abdominal flank of 4-week-old rats (10^6 cells in 100 µl PBS/animal), and tumor growth was followed by measuring the largest and smallest diameters as tumor length (cm) × tumor width (cm).

Low-dose therapy (groups 1-4). Six weeks after tumor cell inoculation, when the tumor nodule had an average size of 5 × 6 mm, treatment with low-dose 5-FU (5 mg/kg body weight) i.p. and/or simultaneous intratumoral AAV-2 infection (10^8 i.u.) was started and repeated weekly. Animals were separated into the following groups: group 1, control, mock-infected (n = 12); group 2, 5-FU (5 mg/kg body weight i.p., n = 8); group 3, AAV-2-infected (1 × 10^8 infectious particles i.t., n = 8); and group 4, AAV-2 + 5-FU (n = 8).

High-dose therapy (groups 5-8). Eight weeks after tumor cell inoculation, when the tumor nodule had an average size of 7 × 9 mm, treatment with high-dose 5-FU (50 mg/kg body weight) i.p. and/or simultaneous intratumoral AAV-2 infection (10^8 i.u.) was started and repeated weekly. Animals were separated into the following groups: group 5, control, mock-infected (n = 12); group 6, 5-FU (50 mg/kg body weight i.p., n = 8); group 7, AAV-2-infected (1 × 10^8 infectious particles i.t., n = 8); and group 8, AAV-2 + 5-FU (n = 8). When the tumor reached >500 mm, animals were killed. Twelve (groups 1-4) or 10 (groups 5-8) cycles of treatment were performed, and tumor size and survival rate were documented weekly.

5. THAT the side effects were evaluated by using the Karnofsky performance index, which evaluates quality of life in humans, and according to recommendations of the Laboratory Animal Science Association, a modified scoring system (Table I, recreated below) was devised to evaluate the clinical performance of tumor-bearing rats 14 weeks after tumor challenge. Automated blood analysis (Technicon H3; Bayer, Leverkusen, Germany) was performed on blood derived from 6 animals of each group using LABfacts Workstation V05.22 software (Bayer).

Table I. Clinical Performance Score

Score	3 points	2 points	1 point
Activity and behavior	Normal	Sluggish or restless	Drowsy or unconscious
Bearing and movement	Normal	Partial limited	Paralyzed or immobile
Nourishment and weight	Normal	Restricted	Emaciated
Hair	Normal	Dull or stand on end	Loss of hair
Pain	No pain	Increased anger on handling	Hunched position, vocalization

Tumor tissues were fixed in 10% neutral formalin or snap-frozen in liquid nitrogen and stored at -80°C. Sections (5 μm) were stained with hematoxylin and eosin using standard procedures.

At least 6 representative tumors of each group of treated or untreated animals were fixed in formalin and analyzed for apoptosis. Immunohistochemic detection and quantification of apoptosis in tumor tissue sections were performed using the *In Situ* Cell Death Detection Kit (Roche, Mannheim, Germany). According to the manufacturer's instructions, sections were stained and analyzed microscopically.

6. THAT our findings confirm the use of AAV-2-mediated sensitization of tumor cells to cytotoxic drugs and demonstrate a clear prevention of chemotherapy-related toxic side effects.

As shown in Table II, setforth below, in the immunocompetent Lewis rats, the combination of intratumoral AAV-2 infection of implanted pancreatic DSL6A tumors with additional systemic 5-FU chemotherapy resulted in significant retardation of tumor growth and prolonged survival, in which the combined effects clearly exceeded those achieved by administration of either agent alone.

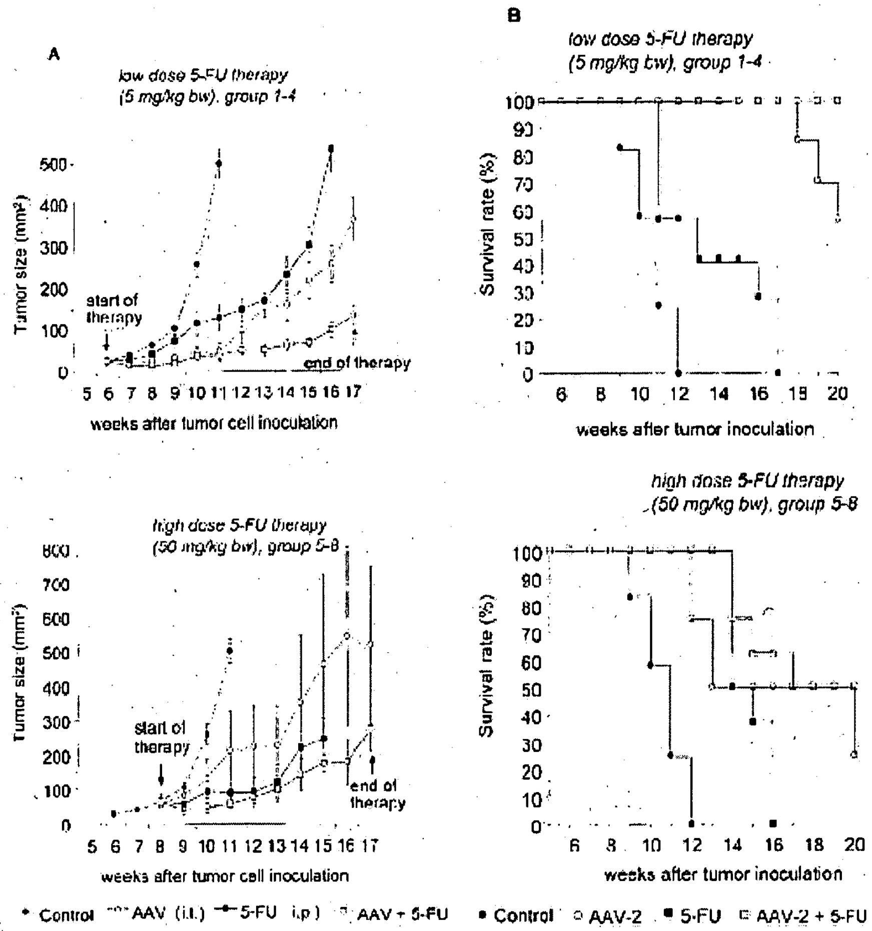
Table II. Survival Rate and Tumor Size of Lewis Rats at the End of Therapy (Week 17 after Tumor Cell Inoculation)

		Control (untreated) (% of control)	**AAV-2 intratumoral (10^8 i.u.) (% of control)**	**5-FU i.p. (% of control)**	**AAV-2 + 5-FU in combination (% of control)**
Groups 1-4 (low dose, tumor size at start: 30 mm^2 for each group)	Survival rate	0/12	8/8	0/8	8/8

		0%	100%	0%	100%
	Tumor size (mm^2)	500[1] ± 35	365 ± 52	533 ± 56	134 ± 22
Groups 5-8 (high dose, tumor size at start: 63 mm^2 for each group)	Survival rate	0/12	4/8	0/8	4/8
		0%	50%	0%	50%
	Tumor size (mm^2)	500[1] ± 35	516 ± 229	244[1] ± 60	221 ± 67

[1] Animals killed earlier due to tumor size or chemotherapy-related side effects.

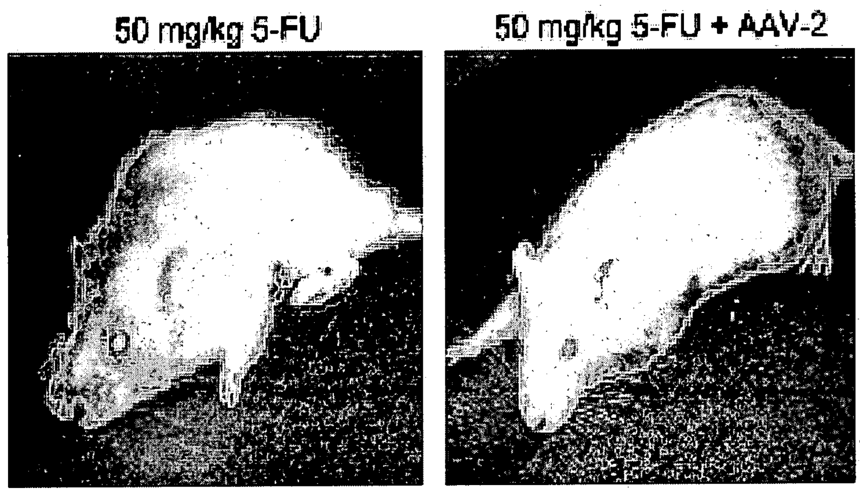
As shown below in Figure 1, in mock-infected rats, tumors grew rapidly (Fig. 1A) and all animals died within 12 weeks after tumor cell inoculation (Fig. 1B). Intraperitoneal 5-FU monotherapy reduced the tumor growth rate, with low-dose 5-FU chemotherapy being less effective at tumor growth inhibition than high-dose 5-FU. However, high-dose 5-FU used as a single agent was highly toxic. All high-dose 5-FU-treated animals died of toxic side effects within 16 weeks (Fig. 1A and Table II), though their tumors had reached only 50% of the size in untreated animals (Fig. 1A, Table II). In rats infected intratumorally with AAV-2 without additional chemotherapy, tumor growth was retarded depending on the tumor size at the beginning of therapy. When AAV-2 treatment was started at a mean tumor size of 30 mm^2, tumors reached a size of 365 (±50) mm^2. In animals in which AAV-2 infection was started 2 weeks later, tumors reached an average size of 516 (±226) mm^2 within 17 weeks after tumor cell inoculation (Fig. 1A). Taken together, neither chemotherapy with different doses of 5-FU nor infection with AAV-2 alone significantly protected rats from tumor progression (Fig. 1A, Table II). In contrast, compared to either treatment separately, tumor growth was most clearly reduced in animals where AAV-2 administration was combined with either low-dose or high-dose 5-FU treatment (Fig. 1A). Three of 8 animals treated with the combined therapy of AAV-2 injection and low-dose 5-FU did not show further tumor progression even 3 weeks after cessation of the treatment.



In general, compared to their uninfected counterparts, all AAV-treated animals, treated with either AAV alone or in combination with 5-FU, showed prolonged survival (Fig. 1B). Although the 17-week survival rate of animals treated with AAV infection alone was the same as that of animals treated with the combination of AAV and 5-FU (Table II), only those animals treated with the combination therapy were still alive 3 weeks after cessation of the treatment (week 20, Fig. 1B, upper panel).

7. THAT in addition to enhancing the chemosensitivity of tumors, AAV-2 treatment resulted in a markedly improved physical condition of the tumor-bearing animals (Fig. 2). To quantify this observation, we applied the a clinical performance score as shown in Table I to evaluate the behavior, movement, hair, body weight and pain as well as hematologic parameters of the animals 14 weeks after tumor cell inoculation and compared the values to those of healthy rats (Table III). These symptoms were accompanied by a 20% decrease in body weight, a dull appearance,

anxious behavior and hunched sitting, which clearly indicated pain (Fig. 2). Concomitant AAV-2 infection, in contrast, provided a striking improvement of the clinical benefit response. In contrast to untreated or 5-FU monotherapy-treated animals, in AAV-infected animals, body weight, hemoglobin and number of white blood cells were similar to those of healthy rats. The most remarkable change of response to high-dose 5-FU therapy when combined with additional intratumoral AAV infection was a rise in the number of monocytes from a mean of 2 cells/μl in 5-FU-treated rats to 1,002/μl, which represents a 4- to 5-fold increase compared to the value in healthy rats (220 cells/μl). In addition, in all AAV-2-treated animals, the number of neutrophil cells was markedly increased.



As shown above in Figure 2, the physical appearance of tumor-bearing Lewis rat on the left after 7 cycles of weekly treatment with only 50 mg/kg 5-FU i.p is in sharp contrast to the animal on the right that received the combination regimen of 50 mg/kg 5-FU and intratumoral infection with MOI 10E8 i.u. AAV-2).

8. THAT in line with previous studies on other cancer entities, our findings confirm the potential use of AAV-2-mediated sensitization of tumor cells to cytotoxic drugs and demonstrate a clear prevention of chemotherapy-related toxic side effects. Furthermore, in immunocompetent rats, combination of intratumoral AAV-2 infection of implanted pancreatic DSL6A tumors with additional systemic 5-FU chemotherapy resulted in significant retardation of tumor growth and prolonged survival, in which the combined effects clearly exceeded those achieved by administration of either agent alone.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States

Code and that such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Magnus Von Knebel-Doeberitz, Ph.D.

Table III. Disease- and Chemotherapy-Related Side Effects

	Body weight (g)	Hemoglobin (dl)	Platelets/l	White blood cells/l	Neutrophil cells/l	Lymphocytes/l	Monocytes/l	Clinical performance score
Healthy rats (normal)	378 ± 24.2	13.5 ± 0.8	621,000 ± 63,200	10,800 ± 887	820 ± 120	9,100 ± 766	220 ± 86	15
	100%	100%	100%	100%	100%	100%	100%	
Untreated (tumor-bearing)	321 ± 33.4	12.7 ± 1.2	536,000 ± 72,000	7,300 ± 1300	111 ± 104	7,180 ± 920	2 ± 25	5
	84%	94%	86%	68%	14%	79%	1%	
AAV-2 (10^8) start size 30 mm^2	372 ± 27.4	14.2 ± 0.9	570,000 ± 57,000	12,400 ± 800	3,918 ± 1,348	7,300 ± 1,207	892 ± 469	12
	98%	105%	92%	115%	478%	80%	405%	
5-FU (5 mg/kg)	355 ± 25.2	13.0 ± 0.7	53,200 ± 75,000	8,843 ± 735	583 ± 468	6,985 ± 963	247 ± 137	6
	94%	96%	9%	82%	57%	77%	112%	
AAV-2 + 5-FU (5 mg/kg)	354 ± 33.6	13.3 ± 1.8	596,000 ± 73,100	11,200 ± 1,040	3,740 ± 1,306	7,134 ± 1,396	616 ± 437	13
	94%	99%	96%	104%	456%	78%	208%	
AAV-2 (10^8) start size 63 mm^2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12
5-FU (50 mg/kg)	306 ± 17.7	6.5 ± 1.4	76,000 ± 55,000	640 ± 520	18 ± 48	606 ± 521	6 ± 68	5
	81%	48%	12%	6%	2%	7%	3%	
AAV-2 + 5-FU (50 mg/kg)	353 ± 22.0	11.6 ± 2.4	540,000 ± 82,800	8,325 ± 790	353 ± 225	6,503 ± 1,324	1,002 ± 336	10
	94%	86%	87%	77%	43%	71%	455%	

Body weight, differential white blood count and physical performance of the animals were determined 14 weeks after tumor challenge. Numbers represent means ± SD of individual values derived from 6 animals of each treatment group. n.d., not determined.